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Pre-Implantation Conceptus and Maternal Uterine Communications: Molecular Events Leading to Successful Implantation

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Abstract. Implantation, a critical step for mammals in establishing pregnancy, requires successful completion of sequential events such as maternal uterine development, conceptus development and attachment, and placental formation. To reach the stage of placental formation, synchronized development of the conceptus and uterus throughout the implantation period is absolutely required. A number of factors expressed at the uterine endometrium and/or conceptus, which are associated with peri-implantation development, have been identified. In addition to a temporal and spatial expression of these factors, their roles in intra- and inter-cellular interactions make it difficult to fully understand physiological roles played during the critical period. This paper focuses on early conceptus development, maternal preparation for implantation and uterine-conceptus communication during the pre-implantation period, rather than the subsequent events such as conceptus attachment to the maternal endometrium. New aspects of pre-implantation processes are evaluated through simultaneous expressions of transcription factors as they possibly regulate the complex processes of implantation events in murine species and ruminant ungulates.
Key words: Pre-implantation period, ICM, Trophoblast, Implantation window, Maternal-conceptus communication
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Unlike those vertebrates that are hatched from eggs, mammals nurture fetuses within the uterus. Beyond egg yolk, mammalian fetuses rely on the continuous supply of maternal nutrients and oxygen, resulting from the maintenance of an intimate relationship through the dynamic structure called the "placenta". Failures in implantation or a lack of sufficient placental development or functioning lead to conceptus losses. Such losses are commonly associated with *in vitro* fertilization procedures in humans and livestock species of the agricultural industry.

Implantation is the process through which a developing conceptus attaches to the maternal endometrium. After successful fertilization, the first step in the peri-implantation period is the migration of the blastocysts from the oviduct into the uterus. As early as 3.5 day post coitus (d.p.c.) in the mouse, the blastocyst differentiates into inner cell mass (ICM) which later forms the embryo, and the trophoblast which later forms the placenta. When a mouse blastocyst reaches the implantation site, it subsequently hatches from the zona pellucida and starts to attach to the endometrium at 4.0–5.0 d.p.c. During this development period, the

uterus in preparation to be receptive to the conceptus is in the critical stage called "implantation window", which is the only time the conceptus can attach to the endometrium. Even before the stage of direct attachment, biochemical communication is thought to exist and function between the uterine endometrium and the conceptus. This maternal-conceptus cross talk influences both pre-attachment conceptus development and attainment of uterine receptivity. Thus, only when the uterus and the conceptus develop in a synchronized manner, the attachment can occur between the trophectoderm cells and endometrium. The maternal-conceptus connection becomes closer when the conceptus invades the endometrium; then early placenta is formed between the uterus and conceptus, and substances vital for survival and further development of the embryo begin to be exchanged through this structure.

Differing from rodents and primates, the ruminant blastocyst undergoes a longer, up to two weeks, pre-attachment period. During this period, ruminants possibly require a longer duration for the establishment of maternal-conceptus communication, which may determine the outcome of implantation. Instead of establishing communication, the processes of active attachment and trophoblast invasion to maternal endometrium may be more important in rodents and primates when compared with that in ruminants. A thesis in this review is based on our hypothesis that conceptuses of ruminants go through closer and more distinct maternal-conceptus communication than those of rodents or primates during the pre-implantation period.

Although the focus of implantation studies generally has been conceptus attachment and invasion to the maternal endometrium, in this paper, the progress of the conceptus and uterus and communication between them in the pre-implantation period are evaluated. This is based on the hypothesis that these events are the starting point of successful implantation. A variety of molecules including adhesion, signaling, transcription, cell cycle and DNA replication proteins coordinate conceptus and uterine development, differentiation and structural formation during this critical phase. In particular, associations and potential interactions among endometrial and conceptus factors are reviewed

and their molecular cascades of expressions during the implantation period are presented.

Pre-implantation Development of the Conceptus

After fertilization in the oviduct, the fertilized ovum moves toward the uterus while undergoing several cell divisions. As the morula reaches the uterus, the first differentiation, formation of the blastocyst occurs. The blastocyst, which contains a blastocoel, consists of two distinct types of cells: the trophectoderm, the outer layer of rapidly dividing cells and ICM, undifferentiated cuboidal cells. The ICM gives rise to the embryo plus extra-embryonic membranes such as allantois and amnion, while the trophectoderm contributes to the trophoblast layers of the placenta. After differentiation to ICM and trophoblast cells, the blastocyst hatches from the zona pellucida and acquires the ability to attach to the uterus. In rodents and primates, blastocysts start attachment and invasion shortly after the hatching whereas in ruminant ungulates, the blastocysts are free floating in the uterus for several days before rapid elongation on days 11 and 12 (day 0=day of estrus). At the end of this elongation the ruminant conceptus occupies almost the entire surface of the uterine luminal epithelia, during which non-specific and loose attachment of the trophoblasts to the endometrium takes place. After going through such physical and biochemical processes of elongation and loose association, the ruminant trophoblasts finally start their attachments to specialized regions, caruncles, of the uterine endometrium.

Transcription factors expressed in ICM or trophoblast lineages

The molecular events underlying the differentiation of distinct cell lineages, ICM and trophoblast, are still not well understood. It is generally believed that transcription factors play critical roles in promoting and driving this first differentiation. Numerous transcription factors are expressed in trophoblasts, including Rex-1 [1], GATA-3 [2], T-box gene Eomesodermin (Eomes) [3, 4], the caudal-related gene Cdx-2 [5], activating protein 2 gamma (AP-2γ) [6, 7], basic helix-loop-helix (bHLH) gene Mash2 [8] and Hand1 [9], and Ets-2 [10]. The T-box gene Eomes and homeobox

gene Cdx-2 are required for early trophoblast development during the pre-implantation period [11, 12]. AP-2γ, Mash2, Hand1 and Ets-2 are involved in trophoblast development during the peri- and post-implantation periods [10, 13–15].

Trophectodermal transcription factors involved in blastocyst formation and implantation

A transcription factor, Eomes, is implicated as an important regulator of gastrulation in *Xenopus* [16], and its expression is also detected during early mouse development [3]. Expression of mouse Eomes is first detected in the trophectoderm cells of the blastocyst, and continues to be found in the extra-embryonic ectoderm of the early post-implantation embryo. Development of mouse conceptuses lacking the Eomes transcription factor is arrested at the blastocyst stage. Morphological analysis at 6.0 and 7.5 d.p.c. revealed that the development of conceptuses with mutated Eomes is arrested soon after implantation and organized embryonic or extra-embryonic structures are not formed. In blastocyst culture, mutant homozygotes did not exhibit trophectoderm outgrowths, although they hatched normally and maintained the typical blastocyst morphology until 7 days after the initiation of *in vitro* culture. In wild type mice, Eomes is only expressed in the trophectoderm during implantation, indicating that Eomes may be required for the differentiation of the trophoblast cell lineage [12].

A transcription factor, Cdx-2, is detected in the epithelium of the adult mouse uterus as one of the homologues of *Drosophila* homeobox gene, *caudal* [17]. In the mouse, immunohistochemical analyses revealed that Cdx-2 is expressed from 3.5 d.p.c. only in the trophectoderm of the blastocyst and later in placental tissues derived from the trophectoderm [5]. Cdx-2 null mutant blastocysts exist in the uterus immediately before implantation (3.5 d.p.c.), but the null mutant blastocyst cannot be detected right after the implantation period (5.5 d.p.c.) indicating that Cdx-2 deficient blastocysts die between 3.5 and 5.5 d.p.c. and fail to implant successfully [11]. Thus, the homozygous mutant mouse conceptuses of Cdx-2 gene show a similar phenotype to that of Eomes, suggesting that Cdx-2 is also necessary for trophectoderm differentiation of blastocysts and for subsequent implantation.

Transcription factor AP-2γ is expressed in the trophectodermal cells prior to implantation and its expression continues in the trophoblast lineages throughout placental development [6, 7]. AP-2γ has been shown to regulate the genes for adenosine deaminase (ADA) [6, 18], human placental lactogen [19] and human chorionic gonadotropin-β [20], which strongly suggests the role of AP-2γ in the regulatory program involving trophoblast cell gene expression. Mutation of the AP-2γ gene in the mouse conceptus shows its necessity in the early post-implantation period since homozygous mutant conceptuses die between 7.5 and 8.5 d.p.c. [21, 22], which is a little later than null mutants of Eomes or Cdx-2 gene. In support of these observations, defective development in embryonic and extra-embryonic structures is noted in histological sections from AP-2γ null mutant mouse conceptuses at 7.5 d.p.c. In addition, *in vitro* culture of the AP-2γ null blastocyst exhibits attachment but defective outgrowth of trophectoderm cells after 7 days in culture [21, 22]. Moreover, in AP-2γ mutant trophoblast cells, Cdx-2 and Eomes expression are significantly reduced or undetectable [21], suggesting that there is a direct association between the function of AP-2γ and the expression of Cdx-2 or Eomes genes in extra-embryonic membranes.

Development in Hand1 deficient mouse conceptuses is arrested by 8.0 d.p.c. with defects in trophoblast cell differentiation and those of Mash-2 die at 10.0 d.p.c. from placental failure [13–15]. Hβ-58 homozygous mutant conceptuses also show a trophoblastic defect from 7.5 d.p.c. and die at about 9.5 d.p.c. [23, 24], though the function of Hβ-58 has not been definitively elucidated. Targeted deletion of the Ets-2 gene causes failure in trophoblast cell migration and differentiation at 7.0 d.p.c. and results in death of the conceptus before 9.0 d.p.c. [10]. While Ets-2 is expressed in many cell types in developing mouse conceptuses [25], it is also expressed in early trophoblast lineage from 7.0 d.p.c. Ets-2 null mouse conceptuses exhibit deficiencies in the expression of the matrix metalloproteinase-9 (MMP-9) gene, whose product is known as a trophoblast derived proteinase that degrades the extracellular matrix and matrices of endometrial epithelial and stromal cells, indicating that Ets-2 might control the degree of trophoblast invasion mediated by MMP-9.

As described above from murine experiments, several genes have been found to be trophoblast specific transcription factors. Different terms in arrested development exhibited by these mutant
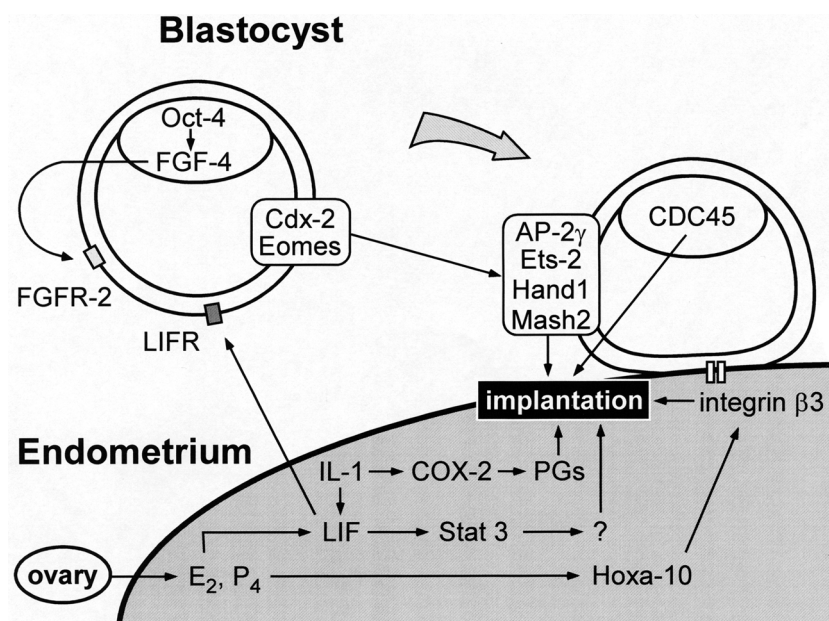
Fig. 1. Gene expression model during the early implantation period in the mouse. In the endometrium, ovarian E_2 induces a cytokine, LIF, which activates Stat 3, resulting in the gene expression cascade required for the uterine preparation for implantation. Serum E_2 and P_4 modulate the transcription factor Hoxa-10 which up-regulates the integrin β3 subunit, a cell adhesion molecule. IL-1 may be involved in PGs production through activation of COX-2 gene. Pre-implantation blastocyst cells differentiate into ICM and trophoblast cells. In ICM, Oct-4 and FGF-4 are required for maintaining pluripotency and there is strong evidence that FGF-4 is induced by Oct-4. FGF-4 also affects the trophoblast cell proliferation through a receptor, FGFR-2. Pre-implantation trophoblast cells express transcription factors such as Cdx-2 and Eomes, which may induce other transcription factors like AP-2, Ets-2, Hand1 and Mash2 involved in various aspects of implantation processes. DNA replication factor CDC45 also influences the degree of ICM cell proliferation in implantation processes.

mouse conceptuses lead us to propose the hypothesis: first transcription factors, Eomes and Cdx-2, influence trophectoderm cell differentiation, followed by sequential expression of transcription factors, Hand1, AP-2γ, Ets-2, Hβ-58 and Mash2, all of which effect development of trophoblastic cells during the early post-implantation period, resulting in the induction of MMP-9 that regulates trophoblast invasion to the maternal endometrium in rodents. Nevertheless, simultaneous expression and interactions of these factors that regulate the trophoblast differentiation and implantation have not been well characterized.

Transcription factors involved in ICM differentiation

Trophoblast cells attach directly to the endometrium; however, ICM is known to be required for the maintenance of trophectoderm, indicating that both ICM and trophectoderm are involved in successful implantation. POU-domain transcription factor Oct-4, essential for the maintenance of pluripotency in the ICM, is expressed throughout oogenesis and pre-implantation development, and its expression is only found in the ICM at the blastocyst stage [26, 27]. The absence of Oct-4 in the mouse conceptus results in peri-implantation lethality from defective ICM development. Because Oct-4 null conceptuses die between 3.5 and 5.5 d.p.c., only traces of trophoblastic cells and decidual swellings are found in the endometrium. Homozygous Oct-4 deficient conceptuses cultured yield only trophoblast cells, but do not contain a recognizable ICM-derived structure. Inactivation of Oct-4 in

embryonic stem (ES) cells reveals that Oct-4 appears to be indispensable for the self-renewing undifferentiated ES cell phenotype. Thus, Oct-4 null conceptuses develop to the blastocyst stage, but the ICM cells are not pluripotent and die, because only the cells of trophoblast lineage are detected. This indicates that Oct-4 is required for early ICM development [28].

In the absence of the ICM, trophoblast proliferation is not maintained in Oct-4 deficient conceptuses due in part to a lack of fibroblast growth factor-4 (FGF-4) expression. FGF-4 is also expressed in the ICM of the murine blastocyst [29, 30]. Analyses of FGF-4 knockout mice, which have phenotypes similar to the Oct-4 mutant, show the requirement of FGF-4 for early post-implantation development [31]. Before implantation (4.0 d.p.c.), FGF-4 null conceptuses exist in the uterus, but after the time of implantation (9.0 d.p.c.) no null mutant embryos exist although the endometrial deciduas are present. In fact, 4.0 d.p.c. FGF-4 null conceptuses appear morphologically normal but histological sections of the uterus show a lack of embryonic structures in FGF-4 null conceptuses at 6.0 and 7.0 d.p.c. FGF-4 null conceptuses cultured display severely impaired proliferation of the ICM, indicating that FGF-4 is essential for ICM development. Thus, both transcription factor Oct-4 and growth factor FGF-4, possibly in coordinated expression, are involved in differentiation and maintenance of ICM. The upstream region of the murine FGF-4 gene contains POU domain to which Oct-4 binds and activates FGF-4 gene transcription that is associated with another transcription factor Sox-2 [32, 33], suggesting that Oct-4 up-regulates FGF-4 expression resulting in ICM differentiation. Furthermore, FGF-4 is not only essential for the maintenance of ICM, but also is critical for trophectoderm proliferation and differentiation. On the other hand, FGF receptor 2 (FGFR-2) is expressed in both ICM and trophectoderm cells [34–36], consistent with a paracrine interaction of the ligand and receptor between ICM and trophectoderm [28, 37]. Null mutation of the FGFR-2 gene exhibits a similar phenotype as FGF-4 mutant conceptuses, which die soon after the time of implantation, resulting from defects in ICM differentiation around 4.5 d.p.c. and onward [38]. FGF-4 has also been shown to be critical in the maintenance of the trophoblast stem (TS) cells *in vitro* [39]. These observations indicate that Oct-4 expressed in ICM of the blastocyst enhances the ICM expression of FGF-4, which affects both ICM and trophoblast development mediated through the FGFR-2 pathway. Thus, the differentiation of ICM and trophoblast cells during the process of implantation is regulated by means of factors like Oct-4, FGF-4 and FGFR-2.

Recently, DNA replication initiator CDC45 deficient mice were shown to be embryonically lethal [40]. CDC45 is an essential gene for the initiation of DNA replication in *Saccharomyces cerevisiae* [41–43]. A human homologue of CDC45 has been isolated, and the encoded protein has been shown to associate with human origin recognition complex (ORC) protein [44] or human minichromosome maintenance protein (MCM) and a subunit of DNA polymerase α [45] in cultured cells. These results indicate that human CDC45 homologue has the same function as yeast CDC45, which also binds to the equivalent proteins and initiates DNA replication. The CDC45 null conceptuses in the uterus disappear soon after implantation between 3.5 d.p.c. and 7.5 d.p.c. CDC45 null blastocysts at 3.5 d.p.c. appear to be normal but *in vitro* culture of such blastocysts shows no proliferation of the ICM after attachment while trophoblast outgrowth appears normal. These results in CDC45 deficient blastocysts are similar to those of Oct-4 or FGF-4 mutant mice, indicating the interrelationship of CDC45 with Oct-4 or FGF-4. Interestingly, mouse conceptuses deficient in several other genes, which are expressed in the cell cycle, such as Chk-1, Brg-1, NEDD-8, and Mat-1, are also reported to die during the implantation period [46–49]. These observations provide a new insight into the importance of cell cycle control in ICM differentiation and implantation processes.

Factors discussed in this section may be studied individually, but the simultaneous expression and interactions of these molecules may be important for elucidating how blastocysts form and develop. It is now apparent that blastocyst formation is regulated by coordinated expression of sets of genes crucial to the progression to successful implantation. More researches focusing on detection of new factors and interactions of the genes so far identified are required to fully understand the implantation processes.

Preparation of the Uterus for
Blastocyst Implantation

Many studies have shown that the uterus during the tightly controlled period called the "implantation window" allows the conceptus to attach to the endometrium in ruminants, rodents and primates. Most mammalian females have estrous cycles during which the endometrium undergoes developmental changes. In the pregnant mouse, the uterus becomes receptive only on 4.5 d.p.c. (day of implantation) and by 5.5 d.p.c., the uterus becomes refractory and fails to respond to the presence of blastocysts. Several endometrial factors controlled by ovarian steroid hormones, estrogen and progesterone, are known to regulate uterine receptivity, resulting in successful implantation. In rodents and primates, the implantation window is established when estrogen affects the endometrium that has been under the influence of progesterone. If estradiol–17β (E_2) levels are kept low during the 24 hours before the conceptus normally attaches, or if progesterone (P_4) is sustained at high levels, the mouse blastocyst remains in diapause (delayed implantation) [50]. Further, an embryo entering the uterus before it has been prepared by a P_4 signal will not implant. Thus, E_2 and P_4 are thought to regulate uterine receptivity directly, though the regulatory mechanisms are not well characterized. The influences of E_2 or P_4 have been examined by deleting the estrogen receptor (ER) or progesterone receptor (PR). Male and female mouse conceptuses with a homozygous deletion of the ER or PR gene developed normally to adulthood, however, female homozygous mutant mice are sterile and show various aspects of reproductive failure [51, 52]. Since ER or PR null conceptuses can survive in the uteri of wild type mice, ovarian steroids do not seem to act directly on conceptuses. Therefore, it appears that some other important molecules, up-regulated through uterine ER or PR, mediate the effect of these steroid hormones, resulting in the establishment of the implantation window.

Transcription factor, Hoxa-10

The homeobox gene, Hoxa-10, null mutant mice show female infertility resulting from defective conceptus implantation [53, 54]. Blastocysts from these mice implant normally when transferred to the uteri of wild-type mice, but blastocysts from wild type mice fail to implant in Hoxa-10 null mice. From these results, Hoxa-10 expressed by the uterine endometrium has been shown to be an essential factor for implantation. Uterine Hoxa-10 is regulated by E_2 and P_4 both in the mouse and the human [55, 56] and high levels of Hoxa-10 mRNA are found in the endometrium during the period of the implantation window [53, 56, 57]. Recently, human uterine Hoxa-10 was demonstrated to induce the expression of uterine epithelial β3 integrin subunit [58], and a lack of its expression could be one possible mechanism by which sterility occurs in Hoxa-10 mutant mice. Conceptus attachment begins from interactions of the blastocyst with the endometrium, which are mediated through cell adhesion molecules such as the integrin family [59, 60]. Integrins, glycoproteins that serve as receptors for extracellular matrix ligands and act as modulators of cellular function, are now one of the best-characterized immunohistochemical markers for uterine receptivity [61]. The β3 integrin subunit exists in the uterine epithelium in the form of αvβ3 integrin, a well-characterized cell adhesion molecule on the luminal surface of the endometrium. Importance of the expression of the β3 integrin subunit gene has been demonstrated by observations in women that aberrant expression of integrins in the endometrium often results in endometriosis, which is associated with infertility due to defective uterine receptivity and implantation failure [62]. From these observations, one possible mechanism in the regulation of implantation is proposed: changes in E_2 and P_4 during the estrous cycle induce Hoxa-10 in the endometrium, which up-regulates the expression of integrins on the endometrial surface, resulting in the attachment of conceptus and uterine endometrium. The product of the homeobox gene Hoxa-11, localized next to the Hoxa-10 gene on chromosome 6, has been shown to possess a function similar to Hoxa-10. In fact, Hoxa-11 mutant female mice are sterile due to their defective uterine environments, since normal litters are obtained when blastocysts from homozygous mutant females are transferred to wild-type mothers [63].

Cytokines

Many cytokines are expressed in the uterus, but only a few have been shown to be required for trophoblast implantation. The clearest example is

the requirement for implantation of maternally produced leukemia inhibitory factor (LIF). In the mouse, LIF has been shown to be expressed in the endometrial glands at the time of blastocyst implantation and is secreted into the uterine lumen [64]. In other mammalian species, including human and ruminants, LIF expression in the uterus is also up-regulated around the onset of blastocyst implantation, suggesting that LIF may be of general significance to blastocyst implantation in mammals [65]. Homozygous LIF mutant mouse blastocysts survive to adulthood, but females are sterile. Null mutant blastocysts, when transferred to the uterus of wild type mice, will implant and develop normally, but the wild type blastocysts do not implant in the uteri of mice with the null mutation in the LIF gene [66]. Thus, endometrial LIF appears to play an essential role in the process of implantation as a factor possibly regulating uterine receptivity. The next question is what regulates LIF expression in the endometrium. Analysis using LIF deficient mice shows that up to the onset of implantation, changes in uterine cell proliferation, hormone levels, blastocyst localization, as well as expression of lactoferrin and Muc-1, do not differ from those in wild-type mice. However, the uterus of the null mutant mouse fails to undergo decidual response to the presence of blastocysts or to artificial stimuli [67]. In rodents, implantation is stimulated by a transient rise in circulating E_2 levels, the nidatory surge, on 4.5 d.p.c. [68, 69]. Nidatory E_2 required for implantation and decidualization in ovariectomized mice could be replaced with uterine expression of LIF [67]. There is still insufficient information on how LIF, up-regulated by E_2 and secreted into uterine lumen, works on the uterus and modulates uterine receptivity and blastocyst implantation. It has so far been shown that LIF acts through the LIF receptor located in the uterine luminal epithelial cells, which activates signal transducer and activator of transcription (Stat) 3 [70]. Thus, one possible mechanism by which E_2 modulates uterine receptivity and implantation is through its up-regulation of endometrial LIF expression and subsequent activation of Stat3. It is thought that LIF is required not only for uterine receptivity, but also for the maintenance of the ICM's pluripotency. LIF was found to inhibit differentiation of mouse ES cells [71, 72]. In addition, ICM from which LIF is removed rapidly loses self-renewal capacity and

the ability to differentiate into a variety of cell types. As both LIF and Oct-4 contribute to the maintenance of the ICM's pluripotency, the potential relationship between these two molecules must be evaluated. Though few experiments have reached definitive conclusions, it appears that effects of LIF and Oct-4 on the retention of cellular pluripotency are exerted in independent pathways [73], and both LIF and Oct-4 are necessary for the maintenance of ICM. Thus, LIF exerts another function as a possible regulator of many important molecules involved in the first cell differentiation of ICM and trophectoderm lineages. These multiple functions of LIF also provide the possible evidence for its role as a maternal-conceptus communication tool: maternally expressed LIF acts on the fertilized ovum and induces its differentiation to form the blastocyst.

Detection of high concentrations of a cytokine, macrophage-colony stimulating factor (M-CSF or CSF-1) in the mouse uterus during pregnancy suggests that M-CSF plays a role outside the hematopoietic system [74]. The synthesis of M-CSF by the uterus, at least during the early part of pregnancy, is regulated by E_2 and P_4 [75, 76]. Results from M-CSF null mutant mice suggest that M-CSF is required for pregnancy, however, the only defect in M-CSF null mice appears to only be smaller litter sizes, indicating that maternal M-CSF may not be an absolute requirement for embryonic survival and development [77].

Interleukin-1 (IL-1) has also been implicated in the process of implantation, since IL-1 mRNA, localized in the peri-implantation mouse uterus, increases from 3.5 d.p.c. and peaks between 4.5 and 5.5 d.p.c. with blastocyst implantation occurring late on 4.5 d.p.c. [78]. IL-1 receptor is also found in the mouse uterus around the time of implantation [79, 80]. In humans, IL-1 and its receptor are detected in the endometrium during the implantation process [81, 82]. Injection of IL-1 receptor antagonist into mice to block the receptor results in blocking of blastocyst attachment to the uterus [83], indicating an important role for IL-1 during the implantation period. However, in the IL-1β gene knockout mouse, reproduction is near normal [84], and mice with the IL-1 receptor deletion are also fertile with a slight reduction in mean litter size [85]. Thus, single deletion of IL-1 or its receptor does not result in implantation defects. Indirect mechanisms by which IL-1 affects

implantation have been evaluated. IL-1 is a potent inducer of LIF expression in cultured human endometrial stromal cells [86]. IL-1 also stimulates prostaglandins (PGs) production by activating cyclooxygenase (COX)-2 expression [87]. PGs have long been speculated to be involved in many aspects of the reproductive functions including implantation and decidualization. COX is an enzyme involved in PG biosynthesis and exists in two isoforms, COX-1 and COX-2. Both COX-1 and COX-2 genes are expressed in the 4.5 d.p.c. mouse uterus, supporting the important roles of COX genes and PGs during the implantation periods [88]. However, COX-1 homozygous mutant females are fertile with limited parturition defects [89], whereas COX-2 deficient female mice are infertile [90]. The COX-2 null mouse shows multiple failures in female reproductive processes including ovulation, fertilization, implantation and decidualization. Further, the implantation defect seen in COX-2 null mice has been demonstrated to be due to a lack of proper production of PGs [91]. Thus, COX-2 expression in the uterus, which is partly stimulated by IL-1, causes increases in PG production that are required for successful implantation. In the uterus, therefore, cytokines appear to interact with each other to regulate various aspects of the implantation process.

Communication between Conceptus and Maternal Uterine Endometrium before Implantation

It is known that the uterus and conceptus initiate and maintain biochemical communications during the pre-implantation period. Numerous observations have revealed that the uterus in the state of "implantation window" allows the conceptus to attach and then invade toward the properly conditioned endometrium, resulting from maternal-conceptus cross talk during the pre-implantation period. In the mouse, LIF receptor or M-CSF receptor is expressed by the conceptuses during the pre-implantation period [92, 93], indicating that LIF or M-CSF is a possible communicator functioning in the maternal-conceptus communication. However, molecular mechanisms by which implantation is established have not been definitively established. This could be due to the fact that the pre-implantation period

is very short and a proper *in vitro* model has not been established. From animal species that have conceptus elongations, maternal-conceptus communication may become more important than in the species such as rodents and primates where the implantation process starts immediately after hatching. The long pre-attachment period during which ruminants' trophoblasts proliferate and elongate extensively has made the maternal-conceptus communication mechanisms more apparent.

In ruminant ungulates such as sheep, cow and goat, a conceptus cytokine, interferon-tau (IFN-τ), is known as a key factor that elicits the process of maternal recognition of pregnancy [94–96]. The expression of IFN-τ is limited to the embryonic trophectoderm during the peri-implantation period [97–100]. The ovine blastocyst begins to secrete IFN-τ immediately after hatching. Its secretion increases markedly when the blastocyst starts to elongate [101] and reaches the maximum level when conceptus attachment to the uterus is initiated on day 16 [97, 102]. The secretion of IFN-τ decreases rapidly as the process of implantation proceeds and when the conceptus is fully attached to the endometrium, IFN-τ is no longer detected [97]. These temporal and spatial expression patterns indicate the existence of an early, trophoblast specific, regulatory mechanism for IFN-τ gene expression.

In mammals, P_4 secreted from the corpus luteum (CL) is necessary to maintain pregnancy. When ruminants do not become pregnant, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) derived from the endometrium induces CL regression and the estrous cycle resumes [103, 104]. Conceptus IFN-τ binds to a type I IFN receptor (IFNAR) located on luminal epithelial cells of the endometrium [105–107], and reduces the numbers of ER and oxytocin receptor (OXY-R) [108–110]. This binding eventually results in the attenuation of endometrial $PGF_{2\alpha}$ secretion, which leads to CL maintenance and continued production of P_4.

While IFN-τ secreted from the conceptus affects the uterine endometrium in a paracrine manner, maternal factors are thought to be essential for the activation of IFN-τ expression. In bovine species, *in vitro* fertilized and cultured blastocysts start to produce IFN-τ without maternal uterine influences. However, if these blastocysts are transferred to the uterus and are again cultured after the recovery,
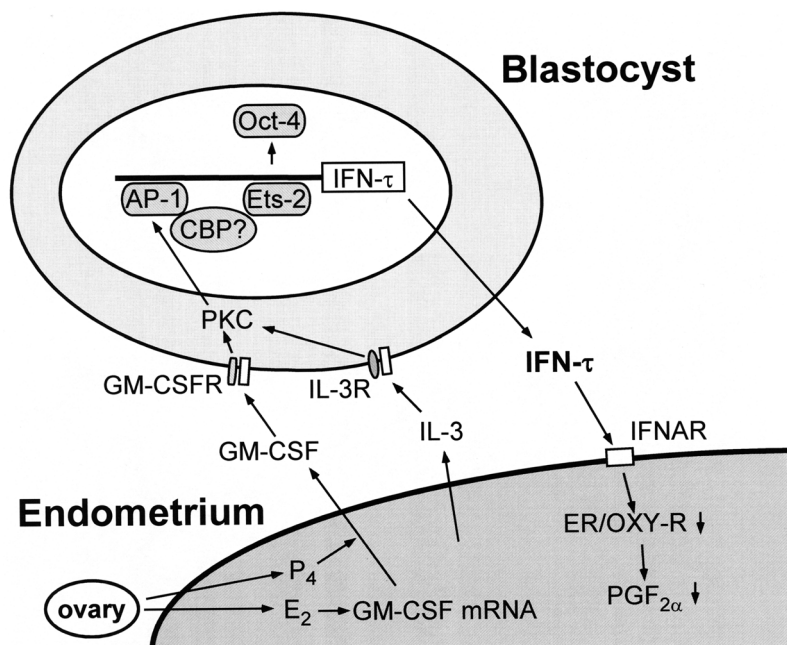
Fig. 2. IFN-τ production resulting from maternal-conceptus communications during early conceptus development in ruminant ungulates. Uterine endometrium derived cytokines, IL-3 and GM-CSF. mRNAs and proteins, are up-regulated by E_2 and P_4, respectively. In the conceptus these cytokines increase expression of IFN-τ through their respective receptors and by intra-cellular signaling through the protein kinase C system (PKC). Transcription factor AP-1 activated by PKC is an important factor that enhances IFN-τ transcription. Another transcription factor Ets-2 is not an effective transcription activator when bound to Oct-4. Once Oct-4 expression subsides or is dissociated from Ets-2, Ets-2 becomes available for IFN-τ gene expression when co-activator CBP connects AP-1 and Ets-2. During the pre-attachment period, conceptus IFN-τ attaches to its endometrial receptor, IFNAR, in a paracrine manner, which reduces the number of estrogen receptor (ER) and possibly oxytocin receptor (OXY-R), resulting in the attenuation of a uterine luteolysin, $PGF_{2\alpha}$. This pathway results in corpus luteum maintenance and assures the continued production of P_4 by the ovary.

they produce approximately 1,000 times more IFN-τ [111], suggesting that IFN-τ production *in vivo* is supported by endometrial factors. Imakawa *et al*. demonstrated that the IFN-τ gene is up-regulated by cytokines, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-3, which are produced by the ovine endometrium and are readily available to the conceptus [112, 113]. Ovarian steroid hormones appear to regulate endometrial expression of GM-CSF: Endometrial GM-CSF mRNA is up-regulated by E_2 and its protein production increases with P_4 treatment [114]. To elucidate how GM-CSF or IL-3 enhances IFN-τ expression, identification of intra-cellular

signaling and/or transcription factors that could regulate IFN-τ expression was required. Several transcription factors, which appear to regulate IFN-τ gene expression, have so far been identified from *in vitro* studies. Transcription factor activated protein 1 (AP-1), a target of an intra-cellular signaling, protein kinase C (PKC), has been shown to enhance IFN-τ expression, resulting from PKC activation by maternal GM-CSF [115, 116]. Ets-2, which often works in collaboration with other transcription factors like AP-1, has also been demonstrated to be an activator of IFN-τ [117]. Considering this with the defective trophoblast function in Ets-2 null mice [10], Ets-2 appears to

play an important role in the trophoblast of many species during the peri-implantation period. Recently, a transcriptional co-activator CREB-binding protein (CBP), has been shown to activate IFN-τ transcription [118]. Because CBP has both AP-1 and Ets-2 binding domains, CBP could be a factor that incorporates effects of AP-1 and Ets-2 on the potentiation of IFN-τ gene transcription. As previously mentioned, mouse embryonic Oct-4, which is expressed only in ICM and not in the trophoblast, is required for ICM differentiation and subsequent ICM development. Oct-4 has recently been suggested to be the repressor for IFN-τ gene regulation since Oct-4 represses IFN-τ transactivation through its binding to Ets-2 in the IFN-τ upstream region [119], fairly consistent with the time- and cell-specific expression of IFN-τ by trophoblast cells.

From these observations, it appears that IFN-τ expression is activated by both AP-1 and Ets-2, however, its expression is usually kept suppressed because experiments on the regulation of IFN-τ expression indicate that the decrease in Oct-4 expression in trophoblast cells removes its suppressive action on IFN-τ gene transcription [119]. Results from ruminant and rodent experimentation suggest that Oct-4 may be repressing many important genes, which are involved in trophoblast differentiation and functions. At the same time, Oct-4, which is known to have both gene activating and gene repressing functions, activates genes such as FGF-4 that is required for ICM maintenance [32, 33]. These gene expressions regulated by Oct-4 may result in differentiation of ICM and trophoblast cell lineages in blastocysts, which lead to the expression of critical factors like IFN-τ that is essential for the implantation process.

Accumulated evidence suggests that IFN-τ is regulated by maternal factors, however, the degree to which the conceptus itself contributes to IFN-τ expression has not been well-characterized. In addition to the activation by transcription factors like AP-1 and Ets-2 [96, 116], conceptus elongation resulting from rapid trophoblast cell proliferation should greatly contribute to IFN-τ secretion. These transcription factors increase IFN-τ production by enhancing the IFN-τ gene transcription of each cell, but the trophoblast elongation also increases IFN-τ secretion due to the proliferation of the number of IFN-τ producing cells. Since DNA replication and cell division are repeated in the cell proliferation process, cell cycle related molecules might also be involved in ruminant trophoblast proliferation leading to IFN-τ activation. Although there is no direct evidence, this hypothesis is supported by experiments with mice in which cell cycle related molecules like CDC45 affect implantation.

Although several factors that regulate IFN-τ production have been detected [96], how IFN-τ exhibits such a specific, temporal and spatial, expression has not been elucidated. Discovery of more factors and their interactions may resolve molecular mechanisms regulating IFN-τ gene expression. Results obtained from such experimentation may then reveal fundamental mechanisms of maternal-conceptus communications beyond the ruminant ungulates, which determine the success or failure of many implantation processes.

Concluding Remarks

To date there have been many reports dealing with biochemical and molecular mechanisms of implantation, but understanding of the regulation of implantation processes is far from being resolved. As discussed in this review, whether or not the blastocysts go through successful implantation may have already begun long before the conceptus attachment to the endometrium. Both pre-implantation conceptus development and uterine preparation including the cross talk of maternal and conceptus tissues should be examined further to fully understand the mechanisms of implantation. The observations that ruminants have a long pre-attachment period and a typical maternal-conceptus cross talk molecule like IFN-τ leads to the hypothesis that ruminants require longer and closer communications between the uterus and conceptus than those of rodents and primates. This characteristic of the implantation process in ruminants should make it possible to understand the mechanisms of maternal-conceptus cross talk that has been difficult to analyze in species like rodents whose pre-implantation period is very short. The processes of implantation represent both unique and common aspects among the species. Mammalian implantation analyses will advance when differences in the characteristics of each species are integrated into such experiments.

In addition, a wider range of molecules, including DNA replication factor CDC45, should be studied further if such factors have roles in the implantation process. These approaches that focus on new aspects of implantation will reveal the associations of all implantation-related factors, which could solve the problems associated with implantation rates in humans and domestic animals.

Acknowledgements

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